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(57) Abstract

New compositions of matter and methods of employing the novel compositions to deliver gene(s) to cells is provided. The compositions of the invention, which comprise a liposomal preparation(s), a nucleic acid molecule(s) and a perpetuation molecule(s) promote the efficient introduction and stable expression of chimeric genes present in the nucleic acid molecule within cells by providing specific mechanisms supporting entry, trafficking, and integration of those molecules within cells. Particular advantages of the invention include greater efficiency and safety than viral methods, combined with stable expression previously obtainable only from viral delivery systems.

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BIOSYNTHETIC VIRUS VECTORS FOR GENE THERAPY

Field of the Invention

The present invention relates to the fields of gene therapy, gene transfer, transgenic organisms and gene expression.

Background of the Invention

Gene therapy involves the introduction of foreign genes into the cells or tissues of a patient in order to treat hereditary, neoplastic, and infectious diseases, such as AIDS. There are two basic methods for the insertion and expression of genes in the cells of higher animals, which are called transfection and transduction. Transfection refers to the use of physical or chemical methods of insertion, which includes microinjection, microparticle bombardment, liposomal preparations, or electroporation, among others. Transduction refers to the use of viral particles or viral mechanisms to gain entry into the cell. The advantage of transfection is that it is relatively safe and efficient - up to 90% or more of transfected cells can express the gene for a limited time. In general, such expression is limited to 2-3 days but can persist for longer periods as well. However, transient transfection techniques have no efficient mechanism for transfected DNA to be maintained in the cell. In contrast, retroviral transduction allows the insertion of genes directly into the genome where they can be maintained, and potentially expressed, for a much longer time. Transduction by certain DNA viruses also allow transduced genes to be replicated in an autonomous fashion, resulting in their perpetuation over a period of time. Unfortunately, transduction of cells by any virus often results in the production of replication-competent viruses, which pose a threat to the host. Moreover, transduction by DNA viruses, as opposed to retroviruses, often requires the expression of one or more viral genes to perpetuate the viral replication process *in vivo*. Viral replication activates the host immune system and results in the elimination or rejection of the virally

transduced cells. Finally, viral expression *in vivo* often decreases or ends altogether within a relatively short time.

The introduction of retrotransposon vectors (see Hodgson, U.S. Patent No. 5,354,674) and synthetic vectors (see WO94/20608) improved the safety and efficiency of gene transfer by removing homology to viral sequences, which improves safety, and by providing a source of transcriptional promoters and enhancers, which improves expression. These improved vectors, however, continued to use retroviral virion-based systems to deliver the genes into cells, which necessitates significant testing of such reagents prior to administering them to humans. Although the risk of viral recombination between the vector and virus-related sequences, which leads to replication-competent virus, was decreased by reducing the homology between the vector and helper sequences to near zero, virion-based delivery systems remain problematic.

In addition to the aforementioned problems, retro-vectors are of little usefulness *in vivo*, because of the rapid inactivation of retroviral particles by serum proteins. Thus, the majority of current gene therapies which employ retroviral virion-based gene transfer must take place in the *ex vivo* setting, i.e., in culture, followed by cell transplantation. Finally, the efficiency of retroviral vector transfer into cells needs to be improved. That is, it would be desirable to increase the efficiency of gene delivery by retroviral helper cells by one to two orders of magnitude, as well as to expand or target the repertoire of cells that can be infected, and to prevent the serum inactivation which takes place *in vivo*.

Various approaches to solving these problems have been attempted. Starting with an inefficient transfection method, i.e., microinjection, Rubin et al. (U.S. Patent No. 4,670,388) delivered a DNA segment containing integration sites recognized by a *Drosophila* derived transposase and another DNA segment which encoded the transposase into cells. They found that the DNA containing the integration sites was inserted into the genomic DNA. A similar method was used to deliver genes into the yeast genome via Ty retrotransposons (Boeke et al., Science, 239, 280

(1988)). Thus, physical methods of delivery can be combined with enzymes encoded by mobile genetic elements to insert genes into cells.

Alternatively, viral delivery and/or expression can be enhanced by physical or chemical transfection methods. For example, Curiel et al. (Proc. Natl. Acad. Sci. USA, 88, 8850 (1991)) mixed inactivated adenovirus particles and DNA and added the mixture to cells. The mixture entered cells by receptor mediated endocytosis. Once inside the endosome, the first vesicle after entrapment, adenovirus proteins associated with the viral capsid disrupted the endosome vesicle in response to acidification of the endosome and ejected the DNA into the cytoplasm.

Synthetic chemical processes have also been used to enhance viral or naked DNA delivery. For example, Wu et al. (WO92/06180) disclose the covalent modification of the surface of retroviruses. The molecule which was covalently bound to the retrovirus binds to receptors on a particular cell surface. Wu et al. also disclose that the injection of a mixture of DNA and poly L-lysine terminated with asialo-orosomucoid resulted in specific uptake and transient expression of the DNA by liver hepatocytes, which have a receptor for asialoglycoprotein. Jolly et al. (WO92/05266) disclose the modification of envelope proteins and other ligands located on the surface of virions to permit the selective entry of those modified proteins and ligands into cells.

Liposomal preparations have been used for drug delivery and as delivery vehicles. Several commercial liposomal preparations are available for the delivery of DNA and RNA to cells for transient expression, as well as for inefficient, stable expression. Such commercial products include Lipofectin™ (Gibco-BRL, Inc., Gaithersberg, MD), Lipofectamine™ (Gibco-BRL, Gaithersberg, MD), and DOTAP™ (Boehringer Mannheim). Usually these commercial preparations consist of various cationic lipid preparations, together with an approximately equimolar amount of neutral lipid. The cationic lipid is thought to interact with the neutral lipid to facilitate charge balancing, lipophilic interactions, and electrostatic attraction and binding to cells, DNA, RNA, and proteins (see Felgner et al., J. Biol. Chem., 269, 2550 (1994);

Debs, WO93/25673). The lipids are usually mixed in an organic solvent, dried under vacuum, and resuspended in aqueous solution by vortexing, homogenization, or sonication prior to use or vending. The aqueous preparations consist of small unilamellar vesicles and large multilamellar vesicles which can be mixed with nucleic acids according to the manufacturer's instructions.

Liposomes can also be modified through the covalent or non-covalent addition of ligands, such as antibodies (Micklus, WO94/02178), which give added specificity to the entry of such liposomal compositions into selective cells. Shoji-Tanaka et al. (Biochem. and Biophys. Res. Commun., 203, 1756 (1994)) reported the liposomal-based delivery of integrase enzyme and a DNA substrate intended for integration into cells. The authors claimed to have achieved specific integration of the substrate DNA at a slightly enhanced rate (3-5 fold) for the liposomal preparation with the integrase and DNA substrate relative to a liposomal preparation of DNA substrate alone. However, the substrate employed by Shoji-Tanaka did not contain the correct recognition signal for integrase, i.e., they employed only one LTR terminus rather than two LTRs, or the correct physical form of the substrate, i.e., they employed a circular DNA substrate rather than a linear DNA substrate.

Lamellar liposomes have been used to infect resistant cells with DNA viruses (Wilson et al., Proc. Natl. Acad. Sci. USA, 74, 3471 (1977)) and retroviruses (Faller et al., J. Virol., 49, 269 (1984)). The encapsulation of a whole virus into lamellar liposomes was a significant breakthrough, although the procedures for entrapment did not significantly improve transduction efficiency (Lasic et al., Science, 267, 1275 (1995)). Sindbis virus nucleocapsids (Kondorosi et al., Biochem. Biophys. Res. Commun., 107, 367 (1982)), as well as poliovirus, have also been encapsulated into lamellar liposomes (Wilson et al., *supra*).

More recently, unilamellar liposomes composed of N-[1-(2,3-dioleyloxy)propyl]-N,N,N-trimethylammonium chloride (DOTMA):cholesterol have been reported to increase the efficiency of infection of CD4+ cells by HIV virus relative to cells exposed to hexadimethrine bromide (polybrene), as

estimated by increased levels of p24 antigen expression at seven days post-infection (Konopka et al., J. Gen. Virol., 71, 2900 (1990)). However, because HIV is an infectious virus, it is impossible to accurately ascertain the efficiency of initial infection by measuring levels of p24 seven days post-infection. In contrast, a similar cationic lipid reagent (DOTMA:dioleylphosphatidylethanolamine [DOPE], a.k.a. Lipofectin™, GIBCO-BRL, Gaithersberg, MD) was used to infect mink cells, which lack an ecotropic viral receptor, with ecotropic retrovirus, although the infection rate was very low (Innes et al., J. Virol., 64, 957 (1990)). It is therefore desirable to improve both the efficiency and safety of liposomal based gene transfer, and in particular to devise processes for doing so with replication-defective vectors of chimeric genes for gene therapy and transgenics.

Summary of the Invention

The present invention provides gene delivery compositions and methods for introducing the claimed compositions into cells. The gene delivery compositions of the invention comprise a liposomal preparation, a nucleic acid molecule, and a perpetuation molecule.

The liposomal preparations of the invention augment or replace functions associated with viral gene transfer. The liposomal preparations of the invention preferably comprise a cationic lipid moiety, preferably a polycationic lipid moiety. Cationic lipid moieties useful in the practice of the invention include, but are not limited to, DOSPA, DMRIE, DOTAP, DOGS, DOTMA, DDAB, L-PE, a starburst dendrimer, or a starburst dendrimer covalently or noncovalently associated with an acyl moiety which can serve as an anchor to a biological membrane (e.g., a diacyl glycerol with a linking moiety, such as phosphatidyl serine). A preferred embodiment of the invention is a cationic lipid moiety selected from the group consisting of DOSPA, DOTAP, DOTMA, DDAB and L-PE. Another embodiment of the invention is a liposomal preparation having a cationic lipid moiety and a neutral lipid moiety. A neutral lipid moiety useful in the practice of the invention includes, but is not limited to, phosphatidyl ethanolamine,

phosphatidyl inositol, dioleylphosphatidyl ethanolamine (DOPE), cholesterol, and their analogs or derivatives. The liposomal preparation of the present invention can be a unilamellar vesicle, a cytofectin, a multilamellar vesicle or other naturally occurring lipid particle.

A preferred embodiment of the invention includes a liposomal preparation comprising a lipid moiety and a cellular protein which facilitates and/or enhances entry or trafficking of the gene delivery composition in the cell, such as a cell receptor ligand, a cell receptor, an antibody, a portion of an antibody, a T cell receptor, a glycoprotein, a chimeric protein and/or an asialoglycoprotein. Protenoids such as PODDS and CADDSYS compounds (U.S. Patent 4,925,673) can also be combined, covalently or noncovalently, with the liposomal preparations to enhance cellular uptake.

The gene delivery compositions of the invention comprise a nucleic acid molecule which comprises a chimeric gene. The nucleic acid molecule of the invention can be associated with at least one purified viral particle. A preferred embodiment of the invention is a gene delivery composition comprising a liposomal preparation and a viral particle, preferably a modified or inactivated viral particle. For example, the viral particle can be inactivated by physical or chemical treatment, or the viral capsid of the viral particle can be modified by partially or completely disrupting the capsid. Moreover, purified viral components can be used in place of any portion of the virus particle. Furthermore, molecules derived from non-viral mobile genetic elements and/or cells can be substituted for such viral components. A purified viral particle useful in the practice of the invention includes, but is not limited to, an enveloped RNA virus, such as a retrovirus, a retrotransposon pseudotyped into a retrovirus, a synthetic retroelement, or other RNA viruses, or a DNA virus, such as a poxvirus, a hepadnavirus, an adenovirus, a herpes virus and the like, or a caulimovirus and the like. The virus is preferably an enveloped virus containing negatively charged phospholipids in its outer shell.

The nucleic acid molecule of the present invention can further comprise integration signals. A preferred embodiment of an integration signal useful in the practice of the invention includes, but is not limited to, retroviral

attachment (att) or integration sites, parvovirus inverted terminal repeats, transposase recognition sites, retrotranpsoson recognition sites, retroelement recognition sites, retrotransposon reverse transcriptase recognition sites, and integrase recognition sites. Examples of retroviral attachment or integration sites include those derived from: Moloney murine leukemia virus, avian sarcoma-leukosis virus, spleen necrosis virus, HIV1, HIV2, reticuloendotheliosis virus, avian myeloblastosis virus, HTLV-1, HTLV-2, lentiviruses, and oncoretroviruses. Other examples of useful integration signals include those recognized by the integrase and transposase enzymes of bacterial transposons, eukaryotic transposons such as *Drosophila* P elements, and the integrative sequences of phages such as Mu, lambda, the phages of *E. coli*, as well as parvovirus inverted terminal repeats.

The gene delivery compositions of the invention further comprise at least one perpetuation molecule, or a nucleic acid molecule which encodes a perpetuation molecule. The perpetuation molecule permits the persistence of the chimeric gene within the cell, either as an element which is integrated into the genome of the cell or through autonomous replication of the gene in the cell. A preferred perpetuation molecule of the invention is a perpetuation protein. A perpetuation protein of the invention includes, but is not limited to, a retroviral reverse transcriptase, an integrase, such as a retroviral integrase, Moloney murine leukemia virus reverse transcriptase, avian myeloblastosis virus reverse transcriptase, avian leukosis-sarcoma virus reverse transcriptase, spleen necrosis virus reverse transcriptase, reticuloendotheliosis virus reverse transcriptase, HIV1 reverse transcriptase, or HIV2 reverse transcriptase, parvovirus rep protein, adenovirus associated virus rep protein, bacterial transposon transposase, bacterial integron integrase, eukaryotic integrase from DNA-based mobile genetic elements and *Drosophila* P element transposase, and other such molecules which permit integration and/or autonomous maintenance of a gene which is co-introduced with the perpetuation molecule within the cell.

Other embodiments of the perpetuation molecule of the invention include nuclear transport proteins or peptides, retroviral matrix

protein, HIV virus matrix protein, nuclear localization signals of the subclasses illustrated by SV40 large T antigen nuclear localization signal, nuclear localization signals of the subclasses illustrated by polycationic polypeptides, poly-ornithine and the like. Yet another embodiment of the perpetuation molecule of the invention is a cellular recombinase such as the *E. coli rec*A protein.

A preferred embodiment of the perpetuation molecule of the invention is a perpetuation protein which is tethered to a peptide or the nucleic acid molecule of the invention to maintain proximity of the peptide or nucleic acid molecule to the perpetuation protein. The tether can comprise a linking molecule, such as an ester, polypeptide, antibody, avidin, streptavidin, biotin, or other molecule attached covalently, or through electrostatic, hydrophobic, ionic, van der waals, affinity, or other chemical interactions, so as to promote maintenance of the tethered complex during entry, transport, integration, and/or maintenance within the cell.

The present invention also provides a method of delivering a gene to a cell which employs the gene delivery compositions described hereinabove.

Brief Description of the Drawings

Figure 1. A. A schematic representation of a gene delivery composition comprising (i) a liposome which comprises cationic lipids, and (ii) an isolated enveloped virus which contains a nucleic acid molecule (genome) and a perpetuation molecule (reverse transcriptase). The positive charge on the lipid neutralizes some or all of the negative charge associated with the viral envelope, increasing the affinity of the envelope glycoprotein or its equivalent for its cognate cellular ligand. B. A schematic representation of a gene delivery composition comprising an isolated virus particle enclosed within a multilamellar liposome. C. A schematic representation of a gene delivery composition comprising (i) a nucleic acid molecule which comprises a therapeutic gene, integration (att) and packaging sequences (Ψ), (ii) a perpetuation molecule, and (iii) a liposome which comprises a targeting

molecule. D. A schematic representation of a gene delivery composition comprising (i) an isolated virus particle, (ii) a liposome comprising cationic lipid, and (iii) DNA segments present on the exterior of the virus particle. Cationic lipids are used to adhere the DNA sequences of interest to the viral particle, while increasing the efficiency of infection of the virus vector. The virus/liposome thus allows the DNA to fuse efficiently to the cell, and in addition, integrase may introduce the sequences into the genome (if the proper *att* signals are present). Viral packaging signals and other response elements may also be present in the DNA to increase its affinity to viral proteins.

Figure 2. A. Left LTR terminus integration signals and consensus sequences from murine retroelements. B. Right LTR terminus integration signals and consensus sequences from murine retroelements. C. Avian myeloblastosis virus attachment/integration sequences. D. Primer sequences incorporating the murine integration sequences, used to make the puromycin resistance vector of shown in Figure 3B2.

Figure 3. A. A schematic representation of lipid and cationic molecules. The dendrimer refers to a prototype, polyamidoamine (PAMAM) cascade polymer, wherein a nitrogen nucleus at the center seeds the aggregation of three additional dendrites, each terminated with a nitrogen that serves as a source of further dendrimer growth. Thus, successive generations of dendrimers arise by sequential addition of units comprising, e.g., CH_2=$CHCO_2CH_3$ (A) and $H_2NCH_2CH_2NH_2$ (B) (generations G0 and G1 are shown). B1. A schematic representation of the BAG vector. B2. A schematic representation of the DNA vector containing the *puro-r* gene. p1 and p2 refer to the primers of Figure 2D, used to amplify the Ψ-SV-puro-r fragment of approximately 1.5 kb. C. Dose response curves for different cytofectins in enhancing efficiency of BAG vector transduction of human HT1080 cells. The number of transduced HT1080 cells (β-gal titer) is shown on the Y-axis, as assayed by direct visualization of β-gal activity (X-gal substrate staining). DOTAP, DOTMA/DOPE and DOSPA/DOPE were used as received from the manufacturers. Concentrations refer to the actual cation concentration, and represent the concentration after application to HT1080 cell

monolayers. Symbols: -V= β-gal titer produced by viral supernatant alone, -P= β-gal titer produced by viral supernatant after pretreatment of HT1080 cell monolayers with 6 μg/ml polybrene. Vertical bars indicate sample standard deviation.

Figure 4. A. Effect of polybrene pretreatment of recipient cells prior to DOSPA:DOPE-mediated transduction. Lane 1, DOSPA:DOPE + virus; Lane 2, DOSPA:DOPE + polybrene pretreatment + virus; Lane 3, polybrene pretreatment + virus; Lane 4, virus alone. The number of transduced cells assayed by direct visualization of β-gal activity (β-gal titer) is shown on the Y-axis. B. Amphotropic and ecotropic virus infection of mouse (NIH3T3= gray bars) or human (HT1080= black bars) cells after DOSPA:DOPE or DOTMA:DOPE treatment of virus. Lanes: 1,2= amphotropic virus alone; 3,4= amphotropic virus + DOSPA:DOPE; 5,6= amphotropic virus + DOTMA:DOPE; 7,8= ecotropic virus alone; 9,10= ecotropic virus + DOSPA:DOPE; 11,12= ecotropic virus + DOTMA:DOPE. C. Effect of treatment of target cells or virus with DOSPA:DOPE. Lane: 1= virus alone; 2= virus treated with DOSPA:DOPE, then added to target cells; 3= target cells treated with DOSPA:DOPE, then virus added. D. Effect of chloroquine (20 mM) on DOSPA:DOPE-enhanced retroviral transduction with amphotropic retrovector. Lane: 1= virus alone; 2= virus + chloroquine (10 minutes), then added to cells; 3= virus + DOSPA:DOPE (20 minutes), then chloroquine added (10 minutes), then added to cells; 4= chloroquine added to cells (30 minutes), then virus added; 5= chloroquine + DOSPA:DOPE added to cells (30 minutes), then virus added; 6= virus + DOSPA:DOPE (30 minutes), then added to cells.

Industrial Applicability

Liposomal preparations carrying various drugs and DNA have been used with success *in vivo* by injection (Debs et al., WO93/25673), and are currently in use for gene therapy clinical trials for nucleic acid delivery both *ex vivo* and *in vivo* (summarized In: The Internet book of Gene Therapy Cancer Therapeutics, Sobol and Scanlon, eds., Appleton and Lange, pp. 283-

296 (1995)). However, in addition to their usefulness in those previous applications, the capability of liposomal preparations to more effectively deliver virus particles and biosynthetic viruses can now be exploited for stable introduction of genes into cells and tissues. Lamellar liposomes (Figure 1B) provide a particulate coat against *in vivo* inactivation of virus by human serum, and provide a possible alternative route for infection (receptor mediated endocytosis). The previous inability of retrovirus capsids to package more than 10 kb nucleic acid molecules can now be overcome by using a lamellar biosynthetic virus (Figure 1C), or by using a virus particle with a nucleic acid molecule which is associated by means of liposomes (Figure 1D). The ability to manipulate the site of attachment and route of entry (endosomal or direct) brought about by the gene delivery compositions of the invention, now makes transductional targeting more feasible. Another important application of the invention is the significant increase (one to two orders of magnitude) in viral titer brought about by cationic liposomal preparations in conjunction with virus (Figure 1A), particularly the polycationic cytofectins. Perhaps the most important contribution of the various embodiments is increased safety of delivery. For cationic enhancement (Figure 1A), a much lower virus dose is required to produce the same effect, limiting patient exposure. For synthetic virus particles (Figure 1C), there is almost no chance for recombination leading to the production of replication virus to occur, since no retrovirus vector is used and no biological entity (i.e., helper cells) is introduced. For DNA pseudotype vectors (Figure 1D) the safety is also enhanced, due to the lack of a retrovirus vector in the vector producer cells. Thus, both the safety and efficiency of gene therapy is enhanced by the various embodiments of the gene delivery compositions of the invention.

Detailed Description of the Invention

The present invention relates to a composition of matter comprising a synthetic lipid (liposomal preparations) together with either virus particles or other materials for the introduction and long-term maintenance of genetic material in host and/or recipient cells, tissues, or organisms. Other

materials which may be included in the composition are nucleic acids, enzymes, ribozymes, proteins, peptide signal sequences, antibodies and the like which are used for helping to introduce the DNA into the genome of the recipient cell. The nucleic acid molecule of the invention may contain recognition signals which are sufficient for integration of the nucleic acid molecule of the invention by means of the enzymes or other materials provided. The nucleic acid molecule may also contain transcriptional promoters, enhancers, matrix attachment regions, locus determining elements, boundary elements and other control elements such as response elements for transcription factors. The nucleic acid molecule may also contain any of a variety of genetic sequences such as protein encoding sequences, ribozyme encoding sequences, antisense, triplex forming regions, introns, exons, etc. intended for therapeutic gene expression.

The liposomal preparations include, but are not limited to, cytofectins, multilamellar vesicles and unilamellar vesicles. A preferred embodiment of the invention is a liposomal preparation which comprises 50% cationic lipid, preferably a polycation such as DOSPA, and 50% neutral lipid, preferably DOPE. Commercially available lipid materials useful in the practice of the invention include Lipofectamine™ (DOSPA:DOPE, Gibco BRL), Lipofectin™ (DOTMA:DOPE, Boehringer Mannheim), and DOTAP™ (Boehringer Mannheim), and the like. Such polycationic lipids greatly enhance transduction efficiency.

The gene delivery compositions of the invention provide a synthetic, virus-like particle, or a composition comprising a virus particle and a liposomal preparation which can neutralize the negative charges associated with biological membranes, such as viral envelopes and plasma membranes. The virus may be modified, for example, by irradiation, e.g., to inactivate nucleic acids, or by partial disruption, e.g., to release enzymes. Ordinarily, the viral particle would adhere to the cell via receptor ligands such as proteins or glycoproteins, leading to uptake of the virus by the cell, sometimes by the process of direct cytoplasmic entry, or in the case of lamellar liposomes, by receptor-mediated endocytosis. By providing a lamellar liposome, cationic

lipid receptors or lipophilic attraction between cationic lipids and anionic phospholipids may substitute for more specific cell-surface receptors found on viruses, thus permitting infection of a broad spectrum of cell types.

Thus, in one embodiment of the invention, a gene delivery composition which comprises a liposomal preparation comprising a cationic lipid moiety, preferably a polycationic moiety, is added to viral particles which comprise a chimeric gene and a perpetuation molecule, or to cells, or both, to neutralize the net negative surface charge on virus and/or cell membranes, so as to enhance the efficiency of infection by at least about a hundred fold or more. The viral particle enters the cell by its normal route, e.g., cytoplasmic fusion, which is restricted to the tropism of the virus.

Yet another embodiment of the invention is a gene delivery composition comprising a lamellar liposomal preparation, preferably a multilamellar liposomal preparation, and a viral particle. The viral particle comprises a nucleic acid molecule, which comprises a chimeric gene, and a perpetuation molecule. This gene delivery composition permits the viral particle to infect cells via endosomal uptake, bypassing the usual tropism of the virus. Furthermore, this gene delivery composition permits the virus to remain unrecognized by inactivating serum proteins, i.e., it is masked from complement recognition by the liposomal enclosure. Gene therapy is a new use for such lamellar liposomes.

In yet another embodiment of the invention, a gene delivery composition of the invention comprises a liposomal preparation, a nucleic acid molecule which comprises a chimeric gene, and at least one perpetuation molecule. A preferred embodiment of the invention includes a linear nucleic acid molecule. A more preferred embodiment of the invention is a linear nucleic acid molecule which comprises sequences which encode integration signals. This embodiment of the invention enhances the safety of gene delivery, because intact viral particles are not delivered, although the advantage of perpetuation is retained. Optionally, this embodiment of the invention can include a number of molecules which can affect the efficiency of gene delivery. Cellular targeting molecule(s) such as an antibody,

protenoid or ligand for a cellular receptor can be attached to the outside of the gene delivery composition, preferably attached to the liposomal preparation. Examples of useful materials that can be combined with the nucleic acid and perpetuation molecule(s) and liposomal preparation include peptide trafficking signals, viral capsid proteins, or intact capsids to permit accurate transport out of the endosome (endosmolytic peptides such as those derived from adenovirus, influenza virus, hemagglutinin and retrovirus capsids, from cationic lipids or protenoids) or into the nucleus (nuclear localization signals), as well as for packaging DNA. For example, polycations such as poly L-lysine or polyornithine can be used to wrap the negatively charged DNA, and to reduce the charge so that it can be packaged more efficiently and compactly by the liposomes. Polycationic materials such as the lysine and ornithine polymers mentioned above can also be used as a means to tether the peptide signals (such as endosmolytic or nuclear localization signals, or other proteinaceous materials thereto attached) to DNA. This is a non-covalent, electrostatic attachment. Integrase or transposase enzyme can also be non-covalently tethered to DNA in this fashion, or it can be covalently attached to the DNA via a linker, such as a peptide, ester, acyl chain, or other covalent linkage. An example of such a linkage is shown in Figure 1C.

In another embodiment of the invention, the chimeric gene(s), which is preferably a linear molecule, more preferably a linear DNA molecule, is combined with a lipid, preferably a polycationic lipid, such as DOSPA:DOPE, and the nucleic acid/lipid complexes are then mixed with enveloped viral particles, preferably retrovirus particles, resulting in viral particles bearing the foreign gene which has been added to the enveloped viral particle by means of the lipid. Upon infection, some of the nucleic acid/lipid complex which is associated with the virus enters the cell along with the virus capsid which contains viral enzymes. Additionally, the cationic lipid may enhance the infectivity of the virus, further aiding efficient penetration of the nucleic acid into the cytoplasm. Furthermore, the DNA may also become integrated via the integrase enzyme associated with the virus if it has terminal *att* (attachment/integration) sites (Figure 2), thus facilitating perpetuation of

the nucleic acid. In addition, the nucleic acid molecule can comprise viral encapsidation signals (Ψ) to enhance the affinity of the entire complex.

Example 1. Cationic lipid/virus

When a cationic lipid, such as a polycationic lipid preparation, e.g., DOSPA:DOPE (Figure 3A), is combined with virus or vector (e.g., the retroviral BAG vector shown in Figure 3A), the transduction efficiency of the vector was greatly enhanced in a dose dependent manner (Figure 3B). The level of transduction, as determined by the β-galactosidase titer of either retrovirus alone (-V) or with 6 μg/ml of hexadimethrine bromide (polybrene, -P) is shown to the left of the graph. The addition of polycationic lipid (Lipofectamine) to the virus enhanced the titer by 1-2 orders of magnitude over the titer of virus alone. The polycationic lipid DOSPA:DOPE was more effective (>60-fold improvement over virus alone) than either DOTMA:DOPE (37-fold) or DOTAP alone (5-fold). The most effective lipid compound, DOSPA:DOPE, is polycationic.

The transduction efficiency of polybrene, a polycation with no lipid moiety, was compared to that of cationic lipids (Figure 3B). Polybrene, although polycationic, was approximately ten times less efficient in promoting transduction than DOSPA:DOPE. Thus, the enhanced transduction efficiency is proportional to the polycationic charge. The membrane anchoring moiety may also contribute to the enhanced transduction efficiency.

To determine whether the observed β-galactosidase expression was transient, or was due to long-term expression brought about by retroviral vector integration, lipid compositions comprising the retroviral BAG vector were mixed with human HT1080 cells. Transduced colonies were selected with the drug G418. Cells treated with Lipofectamine had more than 100-fold more G418 resistant colonies than cells exposed to virus alone, demonstrating that the expression of the transduced gene is associated with integration of the vector which contains the gene.

To determine the effect of a large polycationic charge by itself, in the absence of a membrane anchoring moiety, a molecule known as a

starburst dendrimer, or PAMAM dendrimer (Figure 3A) was added to the virus (Table 1).

Table 1. PAMAM dendrimers: Effect on transduction

Exp. #1: Added to cells		Exp. #2: Added to virus	
dose μg.	blue cells	dose μg.	blue cells
0	0	0	0
0.5	1425	0.5	106
1	1969	1	92
2	510	2	65
3	1895	3	750
4	1070	4	575
5	1458	5	618
6	1225	6	710
7	1118	7	425
8	770	8	405
10	645	10	412
virus	82	virus	146

Sixth generation PAMAM dendrimers were effective at enhancing the efficiency of retroviral transduction by as much as 24 fold over virus alone, and dendrimer treatment of target cells was twice as effective as treatment of virus (see Szoka, WO95/02397; Haensler et al., Bioconjugate Chem., 4, 372 (1993); Tomalina et al., Angew. Chem. Int. Engl., 29, 138 (1990); Redeman et al., J. Cell. Biochem., 21A, 400 (1995)). However, despite their high density of surface charge and greater effectiveness than classical polybrene in enhancing gene transfer, the dendrimers were less effective than a polycation with a membrane anchoring moiety and a neutral lipid (DOSPA:DOPE).

The data shown in Figure 3C indicate the usefulness of combining a polycation (as opposed to a monocation) with a membrane anchoring moiety, thus the combination of a large polycation, such as a starburst dendrimer, with a membrane anchoring moiety, which is either covalently or noncovalently attached, or with a neutral lipid, or both, enhances viral vector transduction. Moreover, large polycations, such as DEAE-dextran or polybrene that were formerly used alone, can be used in combination with lipid moieties, to enhance viral vector transductions. The combination may exert the enhanced transduction effect by stabilizing their complexes with

negatively charged virus particles. Finally, the data shown in Figure 4A demonstrates that the addition of polybrene alone or in combination with lipid, to virus/cells does not further enhance transduction over DOSPA:DPE treatment alone.

If charge neutralization by polycations is the major effector of more efficient transduction, then addition of polybrene to lipid-virus would be expected to have little if any additive effect over and above the more effective cationic lipids. Figure 4A demonstrates the effect of adding polybrene (alone or in combination with lipid) to virus/cells. It is clear that polybrene gives no additional enhancement of transduction over DOSPA:DOPE treatment alone.

Neutralization of the negative charge found on both the cell membrane and the viral envelope may facilitate envelope glycoprotein-receptor interactions in the absence of negative charge repulsion. An alternative explanation for these results is that liposomal preparations can act as direct effectors of receptor-mediated endocytosis via cationic lipid receptors on the cell surface. Cationic lipids would be expected to substitute for the envelope glycoprotein that normally mediates infection of specific cells. Early experiments with lamellar liposomes and retroviruses indicated that infection of non-permissive cells was possible by this route (Faller et al., J. Virol., 49, 269 (1984)), although infection was not very efficient.

Cationic amphiphiles enhance envelope glycoprotein-mediated cellular uptake of retrovirus. To determine whether cationic receptors, which are known to facilitate receptor-mediated endocytosis of lamellar liposomes, could substitute for the retroviral envelope glycoprotein in promoting attachment of virus to cells, the ability of an ecotropic BAG vector derived from the GP+env86 cell line (GP/BAG, capable of infecting murine but not human cells (Markowitz et al., J. Virol., 62, 1120 (1988)) was compared to the BAG vector derived from the amphotropic PA317/BAG helper cell line (PA317 helper cells, Miller et al., Mol. Cell. Biol., 6, 2895 (1986); Price et al., Proc. Natl. Acad. Sci. USA. 84, 156 (1987)), to transduce mouse NIH3T3 fibroblasts and human HT1080 fibrosarcoma cells. Optimal doses of DOSPA:DOPE and DOTMA:DOPE, (Figure 3B) were used to treat virus-

containing supernatants from each vector producer cell line. Figure 4B demonstrates that the normal host range for ecotropic and amphotropic vector producer cells was maintained after DOSPA:DOPE or DOTMA:DOPE treatment of their respective viral progeny, i.e., ecotropic virus infected murine cells whereas amphotropic virus infected both murine and human cells. Two β-gal positive colonies were detected in human cells exposed to ecotropic virus that had been treated with DOTMA:DOPE. Thus, whereas the primary effect of cytofectins was the enhancement of specific envelope glycoprotein-mediated viral infectivity, a lesser degree of cross-infectivity may also be possible, at least with DOTMA:DOPE. Taken together, the data supported the idea that reduction of negative charge repulsion between the cell and viral membrane phospholipids is a mechanism which facilitates the interaction of the envelope glycoprotein and its receptor. Thus, it should be possible to achieve a similar effect by treating the target cells rather than the virus.

Recipient cells, rather than virus, were pretreated with DOSPA:DOPE. This treatment resulted in an enhancement of titer equivalent to, or greater than, that seen when virus alone was treated (Figure 4C). The antimalarial drug, chloroquine, was added in various combinations to the cells, virus, and lipid-virus compositions. Chloroquine is endosmotropic, facilitating the non-degradative route during receptor-mediated endocytosis (Felgner et al., J. Biol. Chem., 269, 2550 (1994)). However, no positive effect resulted from any of the chloroquine treatments (Figure 4D), suggesting that receptor-mediated endocytosis was not the predominant route of uptake. Together these experiments indicated that direct cellular uptake by the normal, envelope glycoprotein-mediated cellular fusion mechanism was responsible for infection, and that, unlike the previous experiments with lamellar liposomes, charge neutralization was the likely mechanism of transduction enhancement.

Example 2. Lamellar liposomes

To determine whether lamellar liposomes enhance viral transduction, virus is introduced into a lamellar liposome, such as a multilamellar liposome. The liposomal preparation may also include other

molecules, which are either covalently or non-covalently attached to the liposomal preparation, such as a receptor or ligand for attachment to specific cells. The gene delivery composition of the invention which comprises the lamellar liposomal preparation may enter cells by receptor-mediated endocytosis, via cationic lipid receptors on the cell surface. The gene delivery composition of this embodiment of the invention prevents inactivation of the virus particle by virus-inactivating complement proteins which are present in human serum. The gene delivery composition of this embodiment of the invention also permits introduction of a vector into cells outside the normal host range of the vector through cationic lipid receptors or by interactions between the cell surface and other molecules associated with the liposomal preparation.

Example 3. A synthetic viral particle

A chimeric nucleic acid molecule is incorporated into a gene delivery composition of the invention by combining the nucleic acid molecule with a retroviral integrase enzyme and a liposomal preparation. The integrase enzyme may be derived from Moloney Murine Leukemia Virus, Spleen Necrosis Virus, Reticuloendotheliosis Virus, Avian Sarcoma-Leukosis Virus, Human Immunodeficiency Viruses 1 and 2, Human T Cell Leukemia Viruses 1 and 2, or Avian Myeloblastosis Virus, among others. A corresponding attachment (*att*=integration) sequence is included on the termini of the nucleic acid molecule. Preferably, the *att* sequence includes two nucleotides which are ordinarily cleaved off the *att* sequence during integrase-mediated integration.

The *att* sequences known to be associated with various integrase enzymes are shown in Figure 2. Preferably, the nucleic acid molecule is a linear DNA molecule such as a cloned fragment or, or a gene amplification product. Preferably, two *att* sites are present per nucleic acid molecule, preferably at the ends of the nucleic acid molecule. Other signals such as packaging signals may be used to selectively enhance the uptake and integration by integrase. Providing a double-stranded DNA molecule as the

nucleic acid molecule eliminates the requirement for reverse transcriptase enzyme activity, other than its associated integrase activity. It may or may not be desirable to include a viral packaging signal, depending upon the specific requirement of the system being used.

Integrase (IN) is located at the carboxy terminus of reverse transcriptase enzymes associated with retroviruses, and is functional either as a reverse transcriptase protein cleavage product or as a part of an intact reverse transcriptase, depending upon the virus or retroelement. Integrase can be made by recombinant DNA methods (Craigie et al., Cell, 6, 829 (1990)), or it can be provided by reverse transcriptase enzyme containing the IN coding region. Reverse transcriptase is also available from commercial suppliers of enzymes, although some commercial products contain IN, while other products have been modified by recombinant DNA technology to eliminate IN activity. Reverse transcriptase and integrase activities can also be isolated from virus by standard methods, or virus preparations can be disrupted to provide these activities, (e.g., by gentle detergent lysis, see Goff et al., J. Virol., 38, 239 (1981)) and the crude preparations can be added to the liposomes.

Each IN protein has its own recognition signal (*att*) at the end of the DNA molecule, which can be determined from the sequence of the cognate virus or retroelement. The recognition signals consist of the inverted terminal repeat at the ends of the long terminal repeats, and occasionally extended sequences beyond the inverted terminal repeat. Usually, from 15-45 bp are required for efficient integration by different viruses. Two of the most commonly available viral enzymes are Moloney Murine Leukemia Virus reverse transcriptase and Avian Myeloblastosis Virus reverse transcriptase. Moloney Murine Leukemia Virus reverse transcriptase can be made by purifying it from virions of cells such as PA317 (available from the American Type Culture Collection (ATCC), Rockville, MD, accession # CRL9078), or any similar cell line producing virus. The virus can be purified by precipitation with ammonium sulfate and concentrated on a sucrose step gradient using the procedure of Fan et al. (J. Mol. Bio., 80, 93 (1973)), as modified by Fujiwara et al. (Proc. Natl. Acad. Sci. USA, 86, 3065 (1989)).

The enzyme extract can be obtained as described by Fujiwara et al., using gentle lysis of the viral particles. A preferred method to obtain integrase is to employ recombinant DNA techniques (Craigie et al., Cell, 6, 829 (1990)). Avian myeloblastosis virus reverse transcriptase can be obtained commercially (from Life Sciences, Inc., St. Petersberg, Florida).

The nucleic acid molecule of the invention comprises a chimeric gene(s) together with the attachment or integration signals recognized by the preferred enzyme (transposase, resolvase, or retroviral integrase). A preferred form of the nucleic acid molecule is DNA, preferably linear DNA, a preferred substrate for integrase. A nuclear acid molecule which contains a chimeric gene(s) of interest is identified, isolated and/or constructed using standard recombinant DNA techniques. The sequences of interest can also be isolated by gene-amplification, by a method such as the polymerase chain reaction, directly from genomic DNA. To amplify a particular sequence primer molecules are constructed that contain sequences complementary to the flanking sequences of the foreign gene of interest, and sequences recognized by IN protein at the termini of each primer.

Alternatively, the integration enzyme may be that of a phage, transposon, insertion element, bacteria or mobile genetic element. Alternatively, the *E. coli* recA protein or a similar recombinase may be combined with the vector in order to increase the efficiency of site-specific recombination. The primers are used to amplify the sequence of interest using a thermal cycler such as the Perkin Elmer Corp. (Emeryville, CA) Tempcycler 2400, or Tempcycler 9600, using an enzyme of great fidelity, such as *Pfu* polymerase, according to the manufacturer's instructions (Stratagene Corp, San Diego, CA). This procedure should result in a linear molecule terminated with the correct attachment/integration sequence for a particular enzyme (for example, see Fig. 2B2). Other enzymes and protocols are available from these two commercial sources that enable the copying of long DNA sequences (>25 kb). The procedure is optimized as to nucleotide concentration, primer concentration, and template, so that the primers are incorporated in an excess of nucleotide after 35 rounds of amplification or less. The amplified fragment

is purified, preferably on an agarose gel, a Qiagen™ column or a spin column, to eliminate excess primers and incomplete product, both of which could otherwise act as competitive inhibitors.

The liposomal transfection procedure is optimized according to the manufacturer's instructions. Next, the enzyme or perpetuation molecule concentration is optimized with respect to the optimum DNA/lipid in an appropriate buffer in the presence, or absence of the necessary divalent cation. The enzyme is added to the nuclear acid molecule at or near 4°C, the enzyme-DNA solution is brought to room temperature, and the solution is combined with the lipid according to the manufacturer's instructions. After incubation, the gene delivery composition comprising the liposomal preparation, DNA and enzyme is added to the recipient cells for up to 24 hours. Preferably, the gene delivery composition comprising the liposomal preparation, DNA and enzyme is added to the cells for at least 5 hours prior to changing the media.

Other molecules may be incorporated into the gene deliver composition of the invention. These molecules include poly L-lysine, poly-ornithine, or other polycations for localization to the nucleus, adenovirus (or fusogenic peptide sequences) for endosmolysis, recombinase proteins to facilitate site-specific recombination and nuclear localization signals for efficient trafficking. Peptide recognition signals can be added to the poly L-lysine chains to facilitate nuclear transport of the nucleic acid molecule. Antibodies or other receptor-like molecules can be attached to the external surface liposome to facilitate delivery. Covalent attachment mechanisms can also be employed to tether the enzyme, DNA, or other molecules in reasonable proximity to each other. Likewise, enzymes can be modified by standard recombinant DNA methods familiar to persons skilled in the art, to include nuclear localization signals, endosmolytic peptides, or to facilitate cell entry, endosmolysis, intracellular trafficking, or entry into the nucleus (see Ausubel et al., Current Protocols in Molecular Biology, vols. 1 and 2, John Wiley and Sons, Inc., 1994, 1995, 1996). An important advantage of this embodiment of the invention is the lack of physical constraint upon the DNA size, due to the lack of a viral capsid. Another advantage is the absence of

opportunities for replication competent retrovirus to form during industrial applications.

Example 4. Nucleic acid pseudotyping

In another embodiment of the invention (Figure 1D), a nucleic acid molecule is combined with a cationic lipid such as DOSPA:DOPE, allowing nucleic acid/lipid complexes to form. The lipid/nucleic acid complexes are then combined with a retroviral supernatant or pellet, forming 3-way complexes with the retroviral particles. Thus, when the virus fuses with the cell, the nucleic acid/lipid complexes are also introduced into the cell. The nucleic acid fragments, e.g., a DNA fragment encoding a protein in which the DNA fragment is preferably terminated with retroviral integration signals, permit the virus to provide the perpetuation molecule, e.g., reverse transcriptase/integrase. The DNA fragment is then integrated more efficiently into the host cell chromosome. The virus may act by facilitating entry of exogenous DNA into the cell by direct cytoplasmic fusion (avoiding the endosome, and possible lysosomal degradation of DNA), or it may facilitate integration via the attachment/integration sequences associated with the DNA fragments. The association of virus, lipid, and DNA enhances the efficiency of long-term transfection/transduction after selection for the foreign gene with a drug such as puromycin (Table 2).

Table 2.
Vector DNA + liposomes + virus

	Exp. #1:			Exp.	Exp.		
Sample:	puro-r	β-gal+	neo-r	puro-r	puro-r	β-gal+	neo-r
(-) control	0	2	0	0	0	0	0
DNA+lip. (250ng)	2	0	0	1	4	0	4
DNA+virus	0	0	5	0	0	9	1
DNA+lip+virus	12	170	45	9	10	345	48
DNA+lip+virus	1	nd	nd	0	2	nd	nd
DNA+lip+virus	6	nd	nd	0	4	nd	nd

A retrovirus vector producer cell line (PA/RVBAG) containing the BAG vector (Figure 3A) was incubated with a mixture of DNA/DOSPA:DOPE. A DNA segment attached to the retrovirus vector as

illustrated in Figure 3A, contains a VL30 encapsidation signal (Hodgson, WO94/20608), a puromycin resistance gene and an SV40 virus early promoter, the murine *att* sequences are at its termini by oligonucleotides during amplification (see Figure 2D). After allowing the DNA/liposomes to associate with the virus, the mixture was permitted to infect human HT1080 fibrosarcoma cells. Controls included no treatment, DNA + liposomes, and DNA + virus. While colonies were occasionally observed after puromycin selection in the wells containing only the DNA fragment and the liposomes (transfection alone), more colonies/ng DNA were always observed in the wells containing the DNA/liposomes/virus mixture, and none were seen in the other control wells.

Duplicate wells were selected with either the drug G418, to determine the *neo* gene titer of the BAG vector contained within the virus, or were stained with X-gal (to determine the comparative β-galactosidase titer of the virus being used). Liposomes increased the *neo* and β-gal titer of the BAG virus. Even in the most dilute samples of DNA/liposomes/virus (1/100 the DNA concentration of the transfected samples, or 2.5ng/well/100,000 cells) yielded puromycin resistant colonies ($>10^6$ puro-r colony/mg). The greater number of puromycin resistant colonies per ng at the highest dilution suggested that higher concentrations of the materials might be toxic or at least suboptimal. Thus, optimization of the procedure with respect to DNA, liposomes, and virus is an important consideration that can vary with the cell type and microenvironment used. While this DNA vector contained a VL30 packaging signal derived from the vector VLPPBN (Hodgson, WO94/20608), another fragment bearing the same genes, but lacking the packaging sequence failed to produce puromycin resistant colonies.

Thus, a DNA segment added to virus particles was efficiently transmitted and expressed, in the absence of retroviral LTRs. Moreover, because the DNA segment is presynthesized and not packaged within the virus capsid, some physical constraints upon vector size are removed. In addition, the lack of oncogenic retroviral LTRs provides an additional margin of safety against remobilization of the vector, replication competent viruses derived by

recombination, and oncogene activation by the LTRs. Although the example employed a vector containing a retrovirus (BAG) as a way to determine the overall effectiveness of the retrovirus particles separate from the puromycin vector, a therapeutic application of this vector would employ helper cell supernatant from a cell line such as PA317 that had not been transfected with a retrovirus vector. The simple DNA vector would be added to the "empty" retrovirus preparation as described herein thus eliminating the retrovirus vector as a substrate for recombination leading to the production of replication competent virus.

The present invention enables those skilled in the art to introduce genes permanently and efficiently into cells using gene delivery compositions of the invention which combine liposomal preparations with viruses or other molecules. The various embodiments of the invention either include biological virus particles or perpetuation materials borne by virus particles, cells or mobile genetic elements. Liposomal preparations of the invention can provide efficient entry of the virus or vector into a recipient cell. If synthetic vectors are employed (Example 3) most vector *cis*-acting sequences, such as long terminal repeats (except *att* sites) can be eliminated. This method has the additional advantage of reducing the exposure of the patient to biological entities, making gene therapy safer and more cost effective.

The invention has been described with reference to various specific and preferred embodiments and will be further described by reference to the following detailed examples. It is understood, however, that there are many extensions, variations, and modifications on the basic theme of the present invention beyond that shown in the examples and description, which are within the spirit and scope of the present invention.

Definitions

The following definitions of biological and genetic terms will be useful in understanding this invention:

DNA segment or sequence: A linear sequence comprised of any combination of the four DNA monomers. The DNA monomers, nucleotides of adenine, guanine, cytosine and thymine code for genetic information, including coding for an amino acid, a promoter, a control or a gene product.

Gene: The smallest, independently functional unit of genetic material which codes for a protein product or controls or affects transcription and comprises at least one DNA segment or sequence.

Chimera: A hybrid gene produced by recombinant DNA technology.

Phenotype: A collection of morphological, physiological and/or biochemical traits possessed by a cell or organism.

Retrotransposon: A cellular, movable genetic element with long terminal repeats.

Vector: Usually an agent transmitting a disease or natural genetic information; here restricted to a genetic agent transmitting a foreign gene (DNA or RNA) construct, unless other indicated.

Genome: One set of chromosomes, haploid or diploid, for an agent or organism.

Transduction: Here limited to the transmission of viral, retrotransposon, or exogenous (added) genes (unless otherwise indicated by means of viral particles or viral functions).

Helper Cell Line: In this context, a cell line which has been genetically engineered or which naturally contains genes capable of generation of some or all necessary retroviral *trans*-acting functions or proteins, such as reverse transcriptase, viral core proteins, envelope glycoproteins, and/or tRNA for priming reverse transcription and the like. Examples of helper cell lines include psi2 and PA317.

Replication Competent Retrovirus (RCR): A retrovirus which bears all genes necessary for *cis* and *trans* functions; complete, able to replicate without additional viral functions.

Non-replication competent (defective) retrovirus: A retrovirus which requires supplemental functions in order to replicate, or which is unable to replicate by itself. In this context, it usually requires *trans* acting functions such as named above.

Transgene: A foreign gene, usually inserted into a vector.

***cis*-acting element:** Genetic element which must be located on the same piece of nucleic acid in order to function, such as transcriptional promoter or enhance elements, primer binding sites and the like.

***trans*-acting element:** Genetic element which need not be located in *cis*, i.e., that which may be located elsewhere, such as in the cellular genome (typically a protein encoding region). Examples of *trans* elements are the retroviral core protein, polymerase, and envelope glycoprotein genes.

VL30: a retrotransposon type: virus-like 30S, found in several species of animals. The DNA consists of long terminal repeats separated by 3-5 kb of internal DNA sequences, which are sometimes expressed in cellular RNA and are packaged into viruses of an infected cell or animal. VL30 genomes tend to lack functional genes in the mouse. VL30 sequences are found integrated at 100-200 copies in the chromosomal DNA of most *Mus* species.

Perpetuation Molecule: a protein, such as an enzyme, or a nucleic acid molecule encoding such an enzyme, which permits the perpetuation of a gene of the invention within a cell, either by the integration of the gene into the host cell genome or through the autonomous replication of the gene within the host cell.

Gene amplification: refers to any of a number of techniques for *in vitro* increasing the copy number of a genetic sequence.

PuXXATG: refers to a translational start codon (ATG) which is preceded three base pairs by a purine base-containing nucleotide, making this ATG a favorable context for the start of translation.

Retro-vector: any vector transmitted using reverse transcriptase to copy an RNA template into DNA (i.e., retrotransposon vectors, retrovirus-derived vectors, synthetic vectors, or retroposon vectors,).

Materials and Methods

Many of the simple recombinant DNA procedures that are not described in detail herein can be obtained by reading a technical reference source such as Ausubel et al., Current Protocols in Molecular Biology, vols. 1 and 2, John Wiley and Sons, Inc., 1994, 1995, 1996.

Cell culture and viral stocks. Vector producer cells (VPCs) PA317/BAG and GP+env86/BAG, producing the retroviral BAG vector were made from PA317 (amphotropic) and GP+env86 (ecotropic) packaging cell lines (cell lines from American Type Culture Collection, (ATCC), Rockville MD, and from Arthur Bank, Columbia University, New York, NY, respectively). Retroviral vector stocks were shown to be replication-defective by marker rescue and amplification assays (Chakraborty et al., Biochem. Biophys. Res. Commun., 209, 677 (1995)). The BAG retroviral vector (provided by Constance Cepko, Harvard University, Cambridge, MA) contains an *E. coli* lac-Z gene (encoding β-galactosidase) expressed from the Moloney murine leukemia virus long terminal repeat and a bacterial neomycin phosphotransferase gene (*neo*) expressed from an internal SV40 virus early gene promoter, thus permitting detection either by β-galactosidase assay or by G418 drug selection. Vector producer cells (VPCs) were grown to near confluence in Dulbecco's modified Eagle's medium (DMEM) from GIBCO-BRL (Gaithersberg, MD) containing 10% fetal bovine serum (FBS). Viral vector supernatant was harvested from VPCs either by centrifugation (3,000 X g, 5 minutes) or by filtration through 0.45 μ m syringe filters (Costar, Cambridge, MA). Target cells used to assay vector infectivity included murine NIH3T3 (ATCC # CRL1658) fibroblast and human HT1080 (ATCC # CCL121) fibrosarcoma cell lines. Both were grown in DMEM supplemented with 10% FBS.

Infection and lipid enhancement procedures.

For transduction procedures, target cells were seeded in multiwell plates at 100,000 per 3 cm tissue culture well on the day before infection, and were maintained in an atmosphere of 95% air/5% CO_2 at 35° C. Centrifuged or filtered viral supernatants (serum-containing medium) were mixed 1:1 with serum-free DMEM media containing varying amounts of Lipofectamine™ (DOSPA:DOPE, GIBCO-BRL) or Lipofectin™ (DOTMA:DOPE, GIBCO-BRL). DOTAP was added according to the manufacturer's instructions (Boehringer-Mannheim, Indianapolis, IN). The virus-lipid mixture was equilibrated at room temperature (22°C) for 30 minutes before being added to target cells. Sterile polystyrene tubes were used exclusively during preincubation of virus and liposomes. In cases where polybrene (final concentration, 6 μg/ml) was used, it was either added to target cells prior to addition of virus-lipid preparations, or else it was added to the virus-lipid preparation at various times as indicated. Medium was aspirated from target cells and was replaced by the virus-lipid preparations (0.5 ml) and supplemented with 2.5 ml of serum-containing medium (3.0 ml, total volume). After 24 hours the medium was changed to DMEM + 10% FBS for an additional 24 hours prior to X-gal staining (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside, from Sigma Chemical Co., St. Louis, MO) or G418 drug selection (600 μg/ml, active G418; GIBCO-BRL). Colonies were counted after 10 days of selection (G418 titer), or individual cells were counted 48 hours post-infection by X-gal staining (β-gal titer). In the case of X-gal staining, blue cells were counted in a 1 mm-wide band across the plate both horizontally and vertically. The cell counts listed reflect the raw data, which can be converted to colony-forming units/ml viral supernatant by multiplying by 141. Error bars indicate sample standard deviation.

Rapid assay for titer enhancement with cationic lipids.

8-16 μl of Lipofectamine was added to 1 ml of serum free medium in a polystyrene tube. 1 ml of viral supernatant (containing serum) was combined with the lipid-containing medium at 20° C for 30-45 minutes. 0.25 ml of the mixture was added to 100,000 cells in a 3 cm tissue culture well, and 2.5 mls

of serum containing medium was added to the cells. After 48 hours, the cells were stained with X-gal as described.

Procedure for using Starburst dendrimers for transduction enhancement. Different concentrations (μg /2 ml/well) of dendrimers as indicated were incubated with either HT1080 cells or viral supernatant for 30 minutes. Briefly, the dendrimers were mixed with 0.25 mls of serum free medium and added to cells, or else it was mixed with 0.25 mls of viral supernatant. The cells were then supplemented to a total volume of 2 mls. After 24 hours, the media was changed to DMEM +10% fetal bovine serum. After another 24 hours, the cells were stained with X-gal to determine the β-galactosidase titer. PAMAM dendrimers were provided by Dr. F. Szoka.

DNA pseudotype vector. DNA was prepared by gene amplification using two oligonucleotide primers as reported in Figure 2 (melting temperature 72° C). The primers contained the murine retroviral *att* sequences found at the termini of U3 and U5 sequences of the retroviral LTR. The gene amplification product contained the entire VL30 packaging sequence of the vector VLPP (Hodgson, WO 94/20608) and the bacterial puromycin resistance gene expressed via the SV40 virus early gene promoter (see vector, Figure 3B2). The polymerase chain reaction (PCR) product was purified by passage through a Worthington spin column to remove residual nucleotides and primers. 8μg of the PCR product was added to 0.5 mls of serum free DMEM media, and 8 μl of Lipofectamine was added to this mixture, which was incubated for 30 minutes. An equal volume of PA/BAG media (containing the retrovirus) was filtered through 0.45 micron syringe filters and added to the liposome/DNA mixture for another 30 min at 25° C. Controls included similar preparations with DNA only, DNA + virus and DNA + liposomes. Various volumes of the reagents were then added to the 3 cm wells and were supplemented with serum containing media to 3 mls. After 24 hours the medium was changed to DMEM + 10% FBS. After another 24 hours, one set of the cells was stained with X-gal to determine the titer of the virus used, while two other sets were selected with either G418 (500μg/ml, 10 days) or puromycin (1μg/ml, 3 days) to determine the number of stably transmitted

recipients. G418 resistance indicated the relative efficiency of the virus vector (BAG) while puromycin resistance indicated the efficiency of the DNA vector.

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(2) INFORMATION FOR SEQ ID NO:1:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 25 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:1:

TGAAGAATAG AAAATTACTG GCCTC
25

(2) INFORMATION FOR SEQ ID NO:2:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 25 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:2:

TGAAGAATGA AAAATTACTG GCCTC
25

(2) INFORMATION FOR SEQ ID NO:3:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 25 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:3:

TGAAGAATAA AAAATTACTG GCCTC
25

(2) INFORMATION FOR SEQ ID NO:4:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 25 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:4:

TGAAGAATAG AAAATTACTG GCCTC
25

(2) INFORMATION FOR SEQ ID NO:5:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 25 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:5:

TGAAAATTAC TGGCCTCTTG TAGAG
25

(2) INFORMATION FOR SEQ ID NO:6:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 25 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:6:

TGAAGAATAA AGATAAAAAA TTACT
25

(2) INFORMATION FOR SEQ ID NO:7:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 25 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:7:

TGAAGAAGGA AAATGAGATT GCCAA
25

(2) INFORMATION FOR SEQ ID NO:8:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 25 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:8:

TGAAGAATGA AAAATTACTG GCCTC
25

(2) INFORMATION FOR SEQ ID NO:9:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 27 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:9:

AATGAAGAAT AAAAAATTAC TGGCCTC
27

(2) INFORMATION FOR SEQ ID NO:10:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 27 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:10:

AATGAAAGAC CCCACCCGTA GGTGGCA
27

(2) INFORMATION FOR SEQ ID NO:11:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 27 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:11:

AATGAAGAAT AAAAAATTAC TGGCCTC
27

(2) INFORMATION FOR SEQ ID NO:12:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 25 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:12:

AGGGTCTCCC CTCGAGGGTC TTTCA
25

(2) INFORMATION FOR SEQ ID NO:13:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 25 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:13:

TGGCCGGGAA TTCGAAAATC TTTCA
25

(2) INFORMATION FOR SEQ ID NO:14:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 25 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:14:

TGGCCGGGAA TTCGAAAATC TTTCA
25

(2) INFORMATION FOR SEQ ID NO:15:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 25 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:15:

AGGGTCTCCC CTCGAGGGTC TTTCA
25

(2) INFORMATION FOR SEQ ID NO:16:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 25 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:16:

AGGGTCTCCT TTTGGGGATC TTTCA
25

(2) INFORMATION FOR SEQ ID NO:17:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 25 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:17:

TGAGGGTCTC CCTTCGGGTC TTTCA
25

(2) INFORMATION FOR SEQ ID NO:18:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 27 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:18:

TGGGTCTCCC CTCGAGGGTC TTTCATT
27

(2) INFORMATION FOR SEQ ID NO:19:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 27 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:19:

GACTACCCAC GACGGGGGTC TTTCATT
27

(2) INFORMATION FOR SEQ ID NO:20:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 27 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:20:

TGGGTCTCCC CTCGAGGGTC TTTCATT
27

(2) INFORMATION FOR SEQ ID NO:21:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 41 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:21:

AATGAAGCCT TCAGCTTCAT TCAGGTGTTC GCAGTCGTCA N
41

(2) INFORMATION FOR SEQ ID NO:22:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 23 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:22:

AATGTAGTCT TAATCGTAGG TTN
23

WHAT IS CLAIMED IS:

1. A gene delivery composition, comprising:
   (i) a liposomal preparation; and
   (ii) a purified virus particle which comprises
      (a) a nucleic acid molecule comprising at least one gene, and
      (b) a perpetuation molecule.

2. A gene delivery composition, comprising:
   (i) a liposomal preparation;
   (ii) a nucleic acid molecule comprising at least one gene; and
   (iii) a perpetuation molecule.

3. A gene delivery composition, comprising:
   (i) a mixture of (a) a nucleic acid molecule which comprises at least one gene, and (b) a liposomal preparation; which is combined with
   (ii) a purified virus particle which comprises a perpetuation molecule.

4. A gene delivery composition, comprising:
   (i) a purified virus particle which comprises (a) a nucleic acid molecule comprising at least one gene, and (b) a perpetuation molecule; and
   (ii) a lamellar liposomal preparation.

5. The gene delivery composition of claim 1, 2, 3, or 4 in which the liposomal preparation comprises a cationic lipid moiety.

6. The gene delivery composition of claim 1, 2, 3, or 4 in which the liposomal preparation comprises a polycationic lipid moiety.

7. The gene delivery composition of claim 1, 2, 3, or 4 in which the liposomal preparation comprises a cationic lipid moiety and a neutral lipid moiety.

8. The gene delivery composition of claim 4 in which the liposomal preparation comprises either small, unilamellar vesicles or large, multilamellar vesicles.

9. The gene delivery composition of claim 1, 2, 3, or 4 in which the perpetuation molecule is a perpetuation protein.

10. The gene delivery composition of claim 9 in which the perpetuation protein is tethered to the nucleic acid molecule.

11. The gene delivery composition of claim 1, 2, 3, or 4 in which the perpetuation molecule is a nucleic acid molecule encoding a perpetuation protein.

12. The gene delivery composition of claim 4 in which the liposomal preparation is a multilamellar vesicle.

13. The gene delivery composition of claim 1, 2, 3, or 4 in which the nucleic acid molecule comprises integration signals recognized by the perpetuation molecule.

14. The gene delivery composition of claim 1, 2, 3, or 4 which further comprises a protein or glycoprotein which specifically binds to a molecule present on cells.

15. The gene delivery composition of claim 14 in which the protein or glycoprotein facilitates entry or trafficking of the composition.

16. The gene delivery composition of claim 1, 2, 3, or 4 in which the nucleic acid molecule is linked to the perpetuation molecule.

17. The gene delivery composition of claim 1, 2, 3, or 4 in which the perpetuation molecule is tethered to the nucleic acid molecule.

18. The gene delivery composition of claim 10, 16 or 17 in which the linking or tethering maintains physical proximity of the perpetuation molecule or protein and the nucleic acid molecule during entry and intracellular transport of the nucleic acid molecule within the cell.

19. The gene delivery composition of claim 1, 3 or 4 in which the viral particle is modified by the addition of DNA or RNA segments.

20. The gene delivery composition of claim 19 in which the DNA or RNA segments encode a gene which is different than the gene encoded by the nucleic acid molecule of claim 1, 2, 3, or 4.

21. The gene delivery composition of claim 1, 2, 3, or 4 in which the liposomal preparation comprises a dendrimer linked to a lipid moiety.

22. A method of introducing a gene into cells, comprising contacting cells with the gene delivery composition of claim 1, 2, 3 4, or 23 so as to yield cells transformed with the gene.

23. A gene delivery composition, comprising:

(i) a dendrimer;

(ii) a nucleic acid molecule comprising at least one gene; and

(iii) a perpetuation molecule.

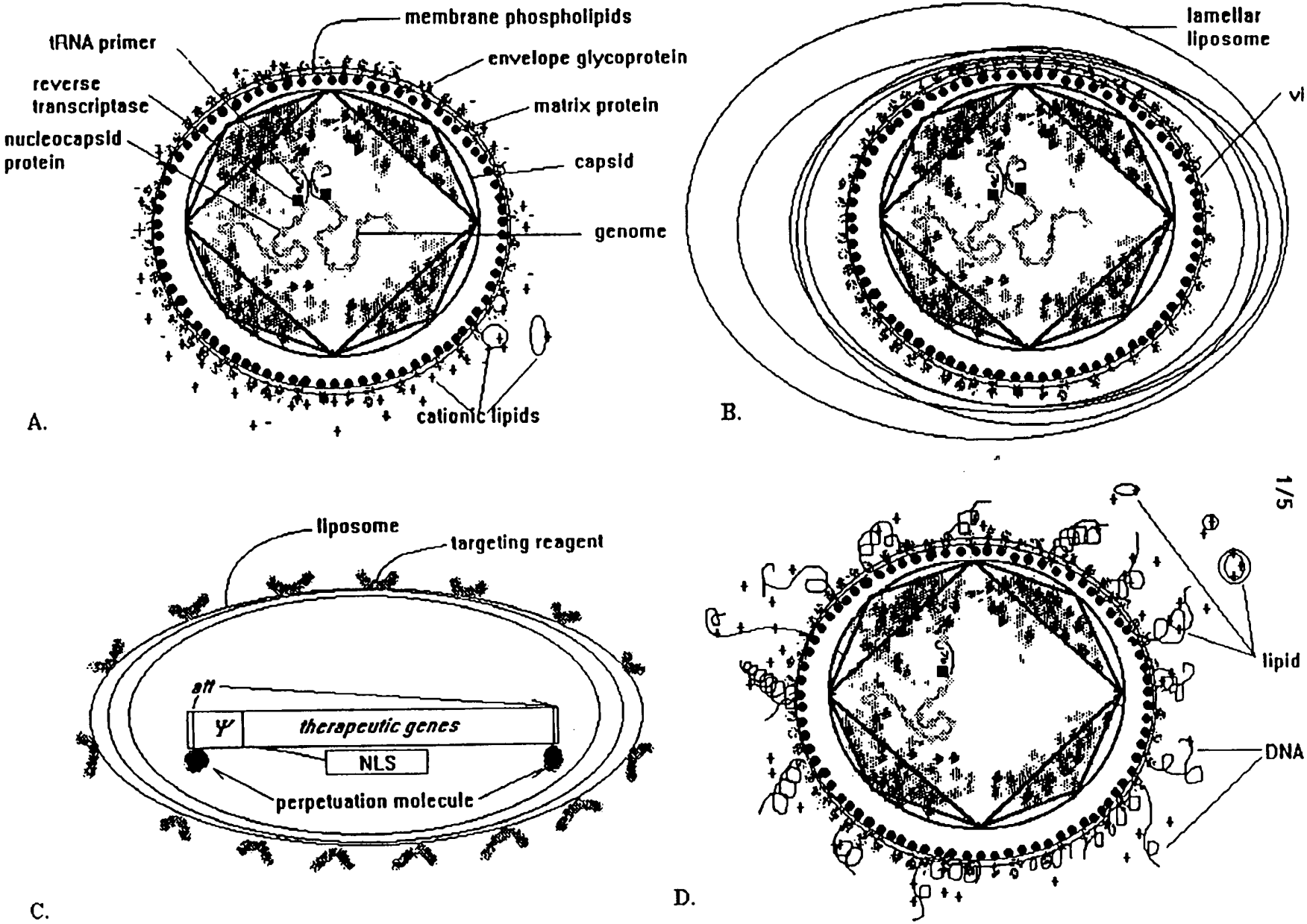

membrane phospholipids
tRNA primer
envelope glycoprotein
reverse transcriptase
matrix protein
nucleocapsid protein
capsid
genome
cationic lipids
A.
lamellar liposome
vi
B.
liposome
targeting reagent
aff
Ψ
therapeutic genes
NLS
perpetuation molecule
C.
lipid
DNA
D.

Figure 1

Fig. 2. Sequences. A. Murine retrovirus concensus left side (U3) integration response (*att*) sequence (reads into LTR). B. Murine retrovirus concensus right side (U5) *att* sequence. C. Avian myeloblastosis *att* sequences (reads toward outside of LTR). D. Primers for amplification of puromycin resistance gene for delivery via murine retrovirus particles.

A.

consensus	**A**	**A**	**T**	**G**	**A**	**A**	**G**	**A**	**A**	T	A	A	A	A	A	A	T	T	A	C	T	G	G	C	C	T	C
							A	**G**	**T**	C	G	G	T	G		C	C	A	C	A	C	T	T	A	G	C	A
										G	C	C	C			C	T	A	G	G	T	A		T	G	A	A	G
																		G		T		G					

B.

consensus	T	G	G	G	T	C	T	C	C	C	C	T	C	G	A	**G**	**G**	**G**	T	C	T	T	T	C	A	T	T
	A	A	A	C	C	G	G	G	A	A	T	C	T	T	G	**A**	**A**	**A**									
	G		C	T	G		C		T	T	G	A			C												
					A																						

C. (left reads into the long terminal repeat, right side reads toward outside of LTR).

left: 5'-AATGAAGCCT TCAGCTTCAT TCAGGTGTTC GCAGTCGTCA...-3'

right: 5'... AACCTACGATTAAGACTACATT-3'

D.

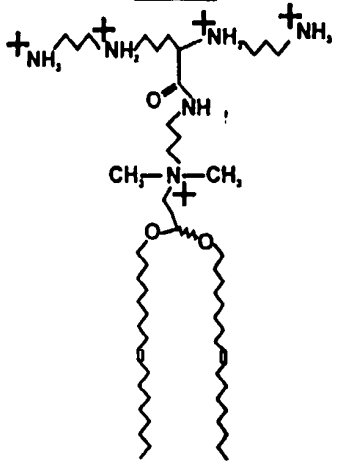
p1: 5'-AATGAAGAATAAAAAATTACTGGTGCATTGGCCGGGAAACAG-3'

p2: 5'-AATGAAAGATTTTCGAATTCTCTAATCTTAGAATTTCAGAAGTCTAGC-3'

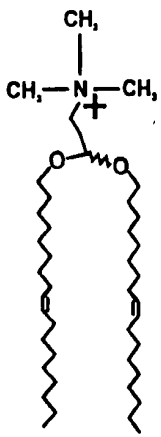
Figure 2

A

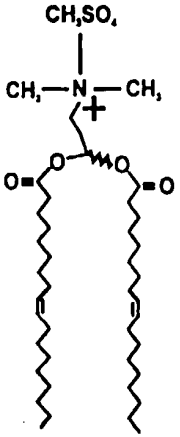
DOSPA



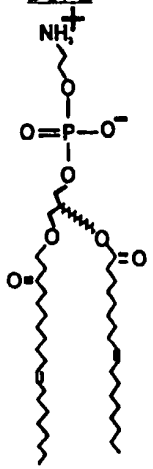
DOTMA



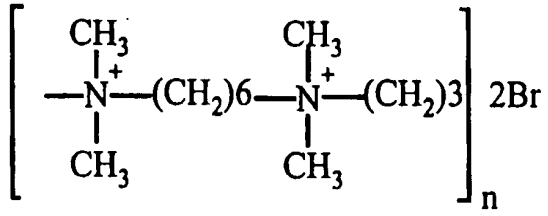
DOTAP



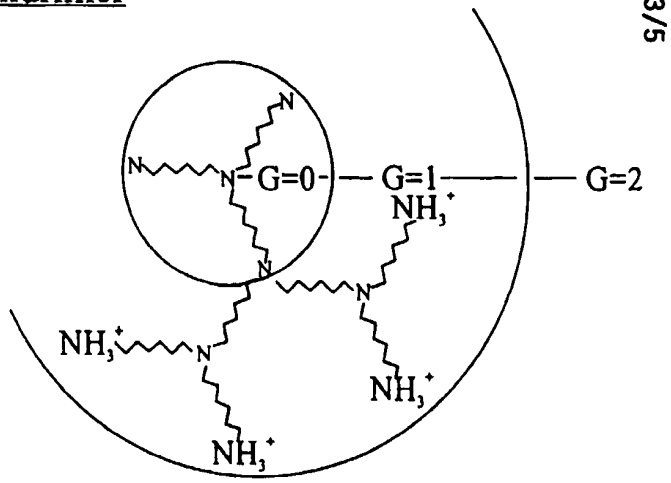
DOPE



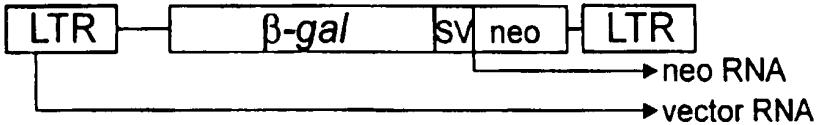
polybrene



dendrimer



B1



B2

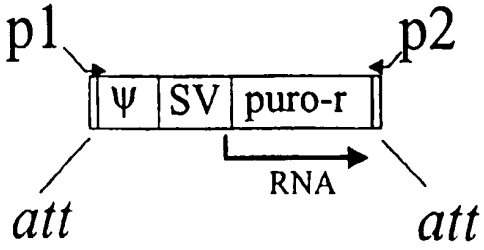


Figure 3A & 3B

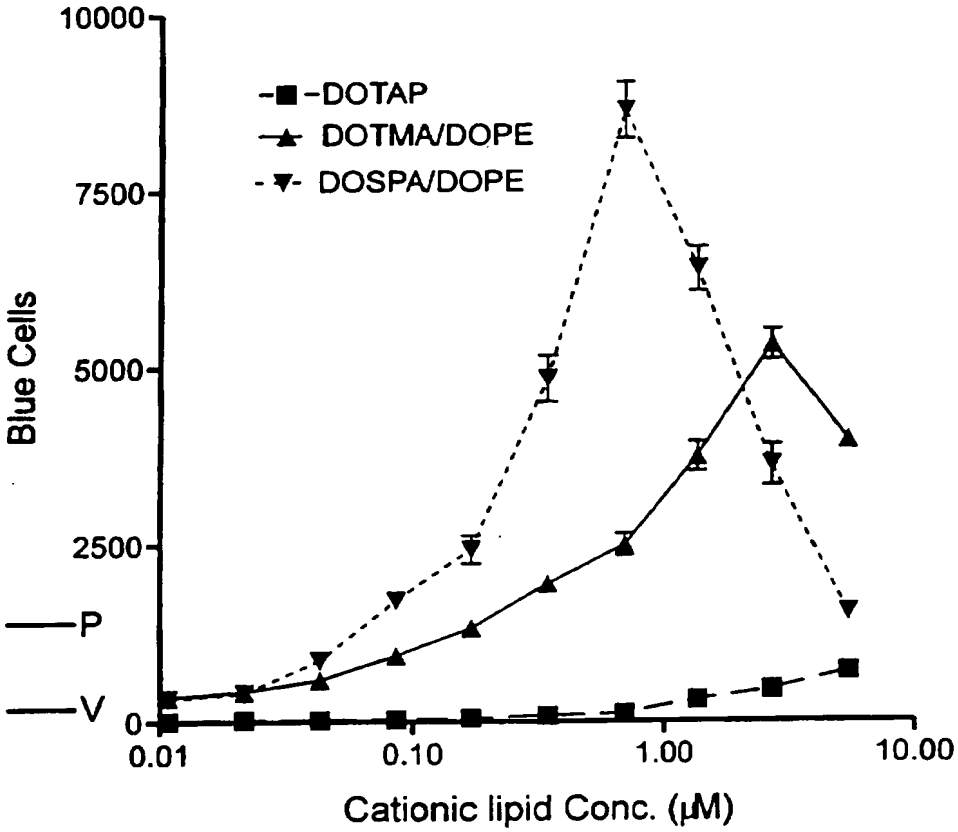

10000
7500
5000
2500
0
Blue Cells
—P
—V
-■-DOTAP
—▲— DOTMA/DOPE
-▼- DOSPA/DOPE
0.01
0.10
1.00
10.00
Cationic lipid Conc. (μM)

Figure 3C

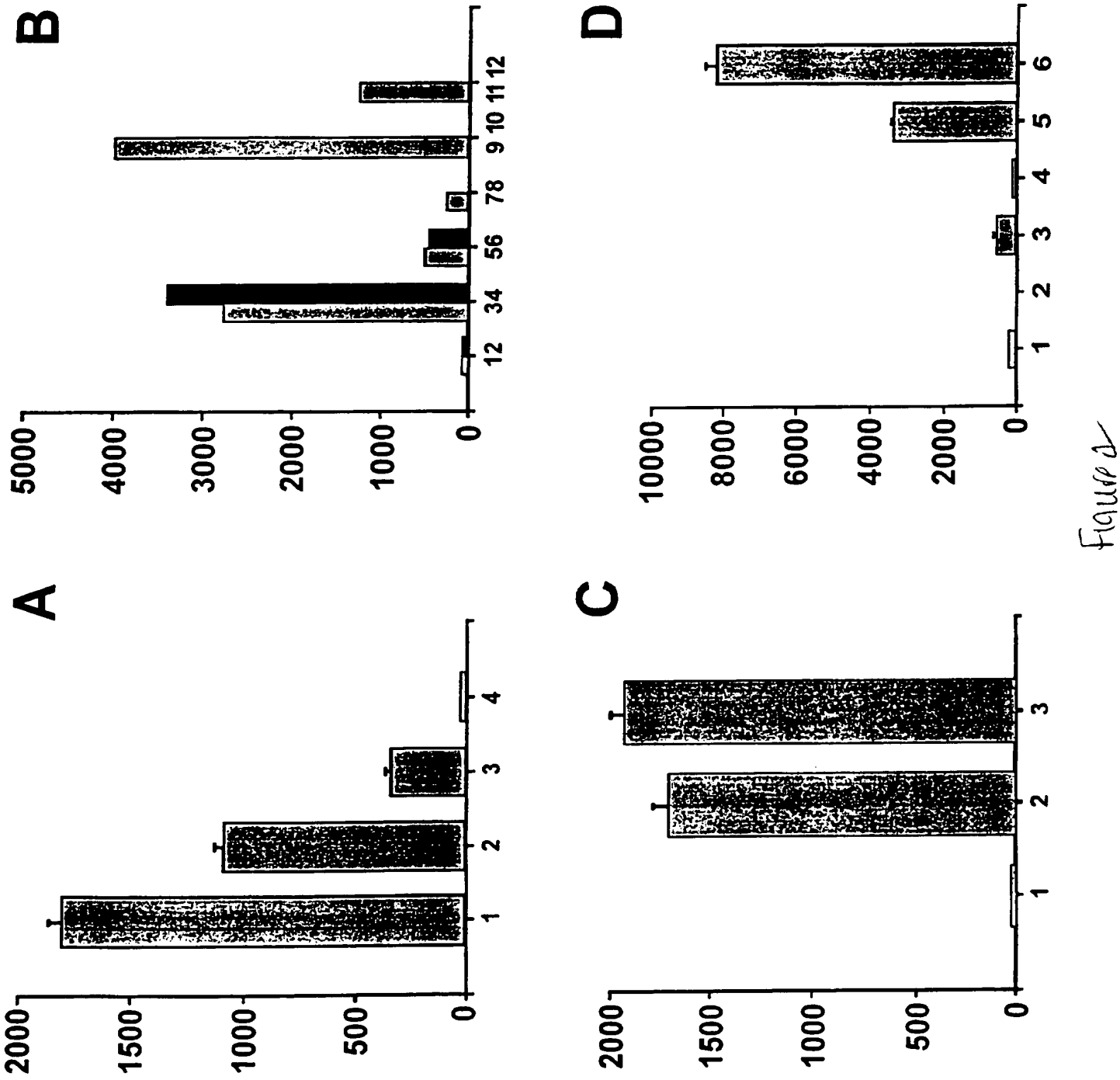

A
2000
1500
1000
500
0
1
2
3
4
B
5000
4000
3000
2000
1000
0
12
34
56
78
9 10 11 12
C
2000
1500
1000
500
0
1
2
3
D
10000
8000
6000
4000
2000
0
1
2
3
4
5
6

Figure 2

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US96/02877

A. CLASSIFICATION OF SUBJECT MATTER
IPC(6) :A61K 48/00; C12N 15/00
US CL : 424/93.1, 93.2; 514/44; 435/172.3
According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 424/93.1, 93.2, 450; 514/44; 435/172.3, 320.1, 935/54

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

APS, CAS ONLINE
search terms: retro(w)vir?, retrovir?, liposom?, dendrimer#, vector?

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X --- Y	INNES et al, Cationic Liposomes (Lifofectin) Mediate Retroviral Infection in the Absence of Specific Receptors, Journal of Virology, February 1990, Vol. 64, No. 2, pages 957-961, see entire document	1-3, 5, 6, 9-11, 16, 17 --- 7, 8, 12-15, 19-21, and 23
Y	US 5,366,737 A (EPPSTEIN et al.) 22 November 1994, column 2.	1-17, 19, 21, 23
Y, P	US 5,470,955 A (CRAIG) 28 November 1995, column 14.	1-17, 19, 21, 23
Y	WO 94/02178 A1 (THE GOVERNMENT OF THE UNITED STATES OF AMERICA) 03 February 1994, see entire document.	1-17, 19, 21, 23

[X] Further documents are listed in the continuation of Box C. [ ] See patent family annex.

*	Special categories of cited documents:	"T"	later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"A"	document defining the general state of the art which is not considered to be part of particular relevance	"X"	document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"E"	earlier document published on or after the international filing date	"Y"	document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"L"	document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	"&"	document member of the same patent family
"O"	document referring to an oral disclosure, use, exhibition or other means		
"P"	document published prior to the international filing date but later than the priority date claimed		

Date of the actual completion of the international search
13 JUNE 1996

Date of mailing of the international search report
18 JUL 1996

Name and mailing address of the ISA/US
Commissioner of Patents and Trademarks
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Authorized officer
JAMES MARTINELL

Telephone No. (703) 308-0196

Form PCT/ISA/210 (second sheet)(July 1992)*

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US96/02877

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X --- Y	WO 93/25673 A1 (THE REGENTS OF THE UNIVERSITY OF CALIFORNIA) 23 December 1993, pages 1-38.	1-17, 19 ---------- 21, 23
Y	US 5,334,761 A (GEBEYEHU et al.) 02 August 1994, entire document.	21, 23

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US96/02877

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This international report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

2. ☐ Claims Nos.:
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

3. ☒ Claims Nos.: 18, 20, 22
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.

2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:

4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

☐ The additional search fees were accompanied by the applicant's protest.

☐ No protest accompanied the payment of additional search fees.

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17
1	**DSM**			**Budget 12/01**			**Sum**	**Status**	**Expected**		**SUM**	**New 100 L Scenario Eurd**			**Status: 20.05.02**		
2				**2001**	**2002**	**2003**		**2001**	**2002**	**2003**		**2002**	**2003**	**Sum**	**2002**	**2003**	**Sum**
3		Assay & Cell Culture Development		1009	557		1566	1243	742	0	1985	742	0	1985	742	0	1985
4		Cell Banking		30	70		100	30	50	0	80	50	0	80	**115**	0	145
5		Scale up	20 L		224		224	0	224	0	224	224	0	224	224	0	224
6			20 L		225		225	0	225	0	225	225	0	225	225	0	225
7			Raw materials		20		20	0	30	0	30	30	0	30	30	0	30
8			50 /100 L		280		280	0	280	0	280	400	0	400	**450**	0	450
9			Raw materials		20		20	0	40	0	40	80	0	80	80	0	80
10			50 L		275		275	0	275	0	275	0	0	0	0	0	0
11			Raw materials		40		40	0	40	0	40	0	0	0	0	0	0
12			50 L		275		275	0	275	0	275	0	0	0	0	0	0
13			Raw materials		25		25	0	40	0	40	0	0	0	0	0	0
14		GMP-run	50 /100 L		684	76	760	0	460	300	760	480	320	800	**580**	**420**	1000
15			Raw materials		30		30	0	40	0	40	80	0	80	**120**	0	120
16		Virus removal study			80		80	0	50	30	80	50	30	80	50	30	80
17		Stability Study				**???**	0		0	0	0	0	0	0	0	0	0
18			**SUM**	**1039**	**2805**	**76**	**3920**	**1273**	**2771**	**330**	**4374**	**2361**	**350**	**3984**	**2616**	**450**	**4339**
19																	
20																	
21	**Octoplus**	Prepayment			10		10		10		10	10		10	10		10
22		Inventarisation			23		23		23		23	23		23	23		23
23		Preformulation			32		32		32		32	32		32	32		32
24		Formulation			35		35		55		55	55		55	**72**		72
25		Filling acute Tox			46		46		46		46	60		60	60		60
26		Filling subacute Tox			41		41		41		41	0		0	0		0
27		Stability Study preclinial			42	20	62		42	20	62	42	20	62	42	20	62
28		Production phase I				35	35			45	45		60	60		60	60
29		stability Study clinical				86	86			86	86		86	86		86	86
30		Project coordination				18	18		33		33	33		33	33		33
31			**SUM**	**0**	**229**	**159**	**388**	**0**	**282**	**151**	**433**	**255**	**166**	**421**	**272**	**166**	**438**
32																	
33		**Overall manufacturing costs in year**		**1039**	**3034**	**235**	**4308**	**1273**	**3053**	**481**	**4807**	**2616**	**516**	**4405**	**2888**	**616**	**4777**
34																	
35		**Budget for Phase II**		**0**	**0**	**2112**	**2112**	**0**	**0**	**2112**	**2112**	**0**	**0**	**0**	**0**	**0**	**0**
36																	
37		**Overall manufacturing costs in year**		**1039**	**3034**	**2347**	**6420**	**1273**	**3053**	**2593**	**6919**	**2616**	**516**	**4405**	**2888**	**616**	**4777**

Zelle: Z1S9
Kommentar: Andreas Herrmann:
Expected values are based on the 50 L scenario as already quoted and considered in the plans

Zelle: Z1S12
Kommentar: Andreas Herrmann:
This scenario allows a phase I study in USA as well as a phase I or II study in europe including a subacute tox-study.
Outcome of a 100 L fermenter will be 550 vials (160 vials acute tox, 100 vials virus removal, 150 vials phase I, remaining vials 140)
Assumption:
Some material from the 1st 100 L run could be pooled with GMP material for subacute tox studies and tox or Phase II study will use not more than 700 vials! (otherwise the amount of 100 L fermenter is not sufficient to perform subacute and phase II study, approx 550 vials available)

Zelle: Z2S14
Kommentar: Andreas Herrmann:
costs for 2001 are included!

Zelle: Z2S17
Kommentar: Andreas Herrmann:
costs for 2001 are included!

Zelle: Z3S8
Kommentar: Andreas Herrmann:
include 333.239,- € (Manhours 11-12/02) and 10848 raw material payed in 20,- €02, but accounted in 2001

Zelle: Z3S9
Kommentar: Andreas Herrmann:
Status: 372000,- € already invoiced
plus additional man-hours during 03/04 approx. 300000,- plus 70000,- € for the retention device
(includes 140000,- € for raw material already paid, i.e. 80000,- € more than expected by DSM)

Zelle: Z4S15
Kommentar: Andreas Herrmann:
additional 65k€ are required for external testing of the MCB at Q-One Biotech!! (not included in DSM budget)

Zelle: Z5S2
Kommentar: Andreas Herrmann:
Prices for Scale up studies are fixed prices. Raw materials will be charged in addition!

Zelle: Z7S9
Kommentar: Andreas Herrmann:
increased costs for medium and column-material based on productivity of cell-line

Zelle: Z8S12
Kommentar: Andreas Herrmann:
I have no quotation for that scenarion so far, thus it is only a reasonable estimation!

Zelle: Z8S15
Kommentar: Andreas Herrmann:
according to quotation from 18.04.02

Zelle: Z9S9
Kommentar: Andreas Herrmann:
increased costs for medium and column-material based on productivity of cell-line

Zelle: Z13S9
Kommentar: Andreas Herrmann:
increased costs for medium and column-material based on productivity of cell-line

Zelle: Z14S2
Kommentar: Andreas Herrmann:
Prices for Scale up studies are fixed prices. Raw materials will be charged in addition!

Zelle: Z14S12
Kommentar: Andreas Herrmann:
I have no quotation for that scenarion so far, thus it is only a reasonable estimation!

Zelle: Z14S13
Kommentar: Andreas Herrmann:
I have no quotation for that scenarion so far, thus it is only a reasonable estimation!

Zelle: Z14S15
Kommentar: Andreas Herrmann:
according to quotation from 18.04.02

Zelle: Z14S16
Kommentar: Andreas Herrmann:
according to quotation from 18.04.02

Zelle: Z15S9
Kommentar: Andreas Herrmann:
increased costs for medium and column-material based on productivity of cell-line

Zelle: Z15S15
Kommentar: Andreas Herrmann:
addtional external costs for characterization of the genetic stability of MCB and EPC

Zelle: Z16S2
Kommentar: Andreas Herrmann:
based on man-hours!!!

Zelle: Z24S15
Kommentar: Andreas Herrmann:

new amendment of octoplus for the extended stability is included (plus 17000,- €)

Zelle: Z25S12
Kommentar: Andreas Herrmann:
estimated!

Zelle: Z25S15
Kommentar: Andreas Herrmann:
estimated

Zelle: Z28S13
Kommentar: Andreas Herrmann:
estimated!

Zelle: Z28S16
Kommentar: Andreas Herrmann:
estimated!

Zelle: Z31S11
Kommentar: Andreas Herrmann:
this budget is already within the status plans (01/02)

Zelle: Z35S2
Kommentar: Andreas Herrmann:
Assumptions taken from original planning. No quotation already available so far!